

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
26 February 2004 (26.02.2004)

PCT

(10) International Publication Number
WO 2004/016779 A1

(51) International Patent Classification⁷: C12N 15/02

(21) International Application Number:
PCT/KR2003/001641

(22) International Filing Date: 14 August 2003 (14.08.2003)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
10-2002-0048683 17 August 2002 (17.08.2002) KR

(71) Applicant and

(72) Inventor: SUH, Hae-Young [KR/KR]; Cheongmyung-maeul Gunyoung Apt. 421-402, Youngtong-dong, Paldal-gu, Suwon-si, Kyungki-do 442-470 (KR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): KIM, Sung-Soo [KR/KR]; Donga-1-cha Apt. 1007, #419-13, Seong-nae-3-dong, Kangdong-gu, Seoul 134-033 (KR). KIM, Ji-Won [KR/KR]; Campusville 702, #38-26, Woncheon-dong, Paldal-gu, Suwon-si, Kyungki-do 442-380 (KR). LEE, Young-Don [KR/KR]; Sunkyoung-3-cha

Apt. 301-504, Ingye-dong, Paldal-gu, Suwon-si, Kyungki-do 442-070 (KR). AHN, Seung-Cheol [KR/KR]; Kwanak Dreamtown Apt. 102-1504, Bongcheon-5-dong, Kwanak-gu, Seoul 151-770 (KR).

(74) Agents: JANG, Seongku et al.; 19th Fl., KEC Building, #275-7, Yangjae-dong, Seocho-ku, Seoul 137-130 (KR).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

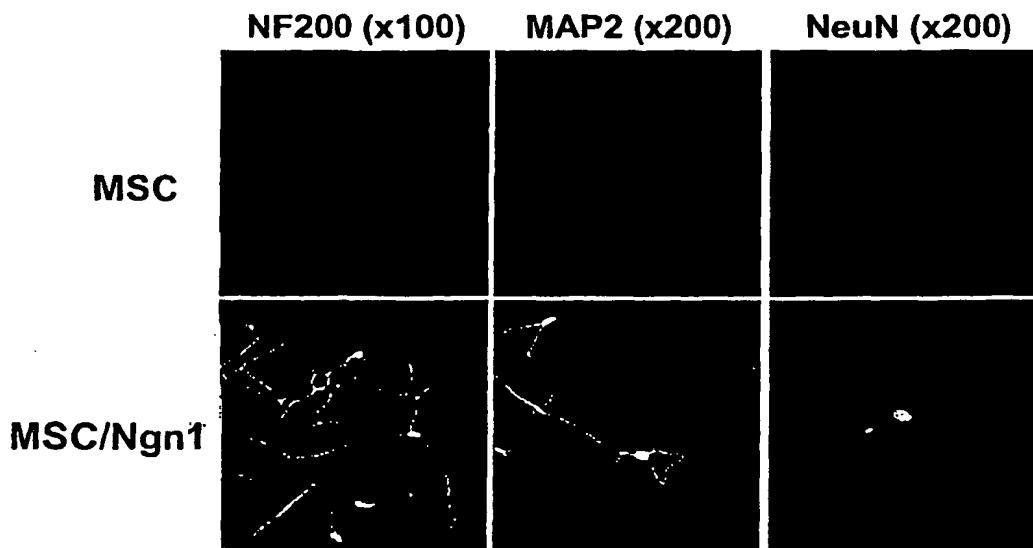
(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

[Continued on next page]

(54) Title: A METHOD FOR TRANSDIFFERENTIATING MESENCHYMAL STEM CELLS INTO NEURONAL CELLS



(57) Abstract: This inventive discloses a method for transdifferentiating mesenchymal stem cells into neuronal cells, which comprises increasing the level of a basic helix-loop-helix (bHLH) transcription factor in the mesenchymal stem cells, said cells being useful in cell therapy or gene therapy for treating brain neurological diseases such as Parkinson's disease, Alzheimer disease, Huntington's disease, amyotrophic lateral sclerosis, cerebral paralysis and brain ischemia; and spine disfunction caused by a traumatic injury.

BEST AVAILABLE COPY

WO 2004/016779 A1



— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

A METHOD FOR TRANSDIFFERENTIATING MESENCHYMAL STEM CELLS INTO NEURONAL CELLS

Field of the Invention

The present invention relates to a method for transdifferentiating mesenchymal stem cells into neuronal cells and a pharmaceutical composition containing the neuronal cells for cell therapy of neurological diseases.

Background of the Invention

Mesenchymal stem cells (MSCs) are multipotent bone marrow stromal cells aiding hematopoiesis and can differentiate into mesodermal lineage cells such as osteocytes, chondrocytes, adipocytes and myocytes. Thus, they can be utilized as sourced for the development of artificial tissues.

It has recently been reported that MSCs have a potential to differentiate into neuroglia in the brain (Azizi., et al., *Proc. Natl. Acad. Sci. USA*, 94, 4080-4085(1998); and Kopen., et. al., *Proc. Natl. Acad. Sci. USA*, 96, 10711-10716(1999)), and therefore, it has been proposed the MSCs can be utilized as sources for the treatment of neurological diseases in the central nervous system(Li et al., *Neurosci. Lett.*, 315, 67-70(2001); and Chen., et al., *Stroke*, 32, 1005-1011(2001)).

Several growth factors or hormones have been known to induce differentiation of MSCs into neuron like cells. However, those methods have the problem of highly producing non-neuronal cells together with neuronal cells (Sanchez-Ramos., et al., *Exp. Neurol.*, 164, 247-256(2000); Woodbury., et al., *J. Neurosci. Res.*, 61, 364-370(2000); and Deng., et al., *Biochem. Biophys. Res. Comm.*, 282, 148-152(2001)). The problem is more pronounced when the cells are transplanted into the brain of experimental animals (Azizi., et al., *Proc. Natl. Acad. Sci. USA*, 94, 4080-4085(1998); and Kopen., et al., *Proc. Natl. Acad. Sci. USA*, 96, 10711-10716(1999)).

Thus, there has existed a need to develop a novel method for higher induction of MSCs into neuronal cells.

Neurogenin and neuroD are the basic helix-loop-helix (bHLH) transcription factors which play important roles in the formation of the nervous system. They form heterodimers with other bHLH transcription factors, such

as E12 or E47 and bind to DNAs containing E-box (CANNTG), or on rare occasions, DNAs containing N-box. Such binding to DNA has been reported to be an essential step for the bHLH transcription factors to induce expression of tissue-specific genes (Lee, *Curr. Opin. Genet. Dev.*, 7, 13-20(1997)).

5 The present inventors have endeavored to develop an effective method for transdifferentiating MSCs into neuronal cells; and have unexpectedly found that MSCs transduced with bHLH transcription factors such as neurogenin and neuroD can continuously express the bHLH transcription factors; and that the MSCs expressing the bHLH transcription
10 factors can be transdifferentiated into a high level of neuronal cells when MSCs are transplanted into the brain of experimental animals.

Summary of the Invention

15 Accordingly, it is a primary object of the present invention to provide a method for transdifferentiating mesenchymal stem cells into neuronal cells.

It is another object of the present invention to provide a pharmaceutical composition for cell therapy of neurological diseases.

20 It is still another object of the present invention to provide a kit for transdifferentiation of the mesenchymal stem cells into neuronal cells.

In accordance with one aspect of the present invention, there is provided a method for transdifferentiating mesenchymal stem cells into neuronal cells, which comprises increasing the level of neurogenic basic Helix-Loop-Helix (bHLH) transcription factors in the mesenchymal stem cells.

25 In accordance with another aspect of the present invention, there is provided a pharmaceutical composition for cell therapy of neurological diseases, which comprises a therapeutically effective amount of MSCs transduced with neurogenic bHLH transcription factors or neuronal cells transdifferentiated therefrom.

30 In accordance with still another aspect of the present invention, there is provided a kit for transdifferentiation of mesenchymal stem cells into neuronal cells, which comprises expression vectors containing genes encoding neurogenic bHLH transcription factors or active fragments thereof.

Brief Description of the Drawings

The above and other objects and features of the present invention will

become clear from the following description of the invention taken in conjunction with the following accompanying drawings, which respectively show:

5 Fig. 1 : light microscopic photographs of mesenchymal stem cells isolated from human bone marrow after cultivating for 1, 2, 3, and 7days *in vitro*;

 Figs. 2A to 2D : photographs showing multi-potentials of MSCs (A: adipocytes stained with oil red-O; B: chondrocytes stained with alcian blue; 10 C: alkaline phosphatase activity in osteocytes; and D: osteocytes stained with von Kossa;

 Fig. 3 : the construction of a retrovirus vector which expresses 15 neurogenin1 (ngn1), a neurogenic transcription factor;

 Fig. 4 : western blotting analysis showing expression of ngn1 in the cytoplasm and nucleus of 293T cells transfected with a retroviral plasmid encoding the ngn1 gene; 20

 Fig. 5 : western blotting analysis showing expression of ngn1 in the cytoplasm and nucleus of MSCs transduced with a retroviral vector encoding the ngn1 gene;

25 Fig. 6 : *in vitro* immunocytochemical staining showing neuronal differentiation of MSCs transduced with a retroviral vector encoding ngn1 using antibodies against NF200 (neurofilament 200), MAP2 (microtubul-associated protein 2) and NeuN (neuronal nuclei) as neuronal markers;

30 Fig. 7 : *in vitro* electrophysiological recordings showing that MSCs transduced with a retroviral vector encoding the ngn1 gene acquired a neuronal phenotype;

 Fig. 8 : *in vitro* western blotting analysis showing neuronal 35 differentiation that MSCs transduced with ngn1/E47 and neuroD/E47 using antibodies against NFM (neurofilament-M), NF200 (neurofilament 200), and β -tubulin III;

Fig. 9 : a fluorescent microscopic photograph showing the integration of MSC/Ngn1 and MSC/lacZ in the rat striatum 2 weeks after transplantation;

5

Fig. 10 : double immunohistochemical stainings for the co-localization of β -galactosidase either with a neuron specific marker, NeuN, or an astrocyte specific marker, GFAP, in the rat brain 2 weeks after transplantation of MSC/Ngn1 and MSC/lacZ;

10

Fig. 11 : double immunohistochemical stainings showing expression of β -galactosidase and a neuron specific marker, NeuN in the rat brain 6 weeks after transplantation of MSC/Ngn1 and MSC/lacZ;

15

Fig. 12 : a graph summarizing the results of Fig. 10; and

Fig. 13 : double immunohistochemical stainings showing co-localization β -galactosidase and Human nuclei in the rat brain 2 weeks after transplantation MSC/Ngn1 (A) and presence of a human specific mature neuronal marker, Tau, using an anti-human Tau antibody (B).

20

Detailed Description of the Invention

In the present invention, the cDNAs of bHLH transcription factors can be prepared by cloning or synthesizing based on the sequence information, including neurogenin 1 gene (Genbank Accession No.: U63842, U67776), neurogenin 2 gene (Genbank Accession No.: U76207, AF303001), neuro D1 gene (Genbank Accession No.: U24679, AB018693), MASH1 gene (Genbank Accession No.: M95603, L08424), MATH3 gene (Genbank Accession No. :D85845) and E47 gene (Genbank Accession No.: M65214, AF352579) in accordance with a conventional method well known in the art. Further, the neurogenic transcription factors which may be used in the present invention include those genes modified by changing, deleting or substituting portion of the genes without any substantial change of the activity of said genes.

35

The mesenchymal stem cells which may be used in the present invention can be isolated from the bone marrow, peripheral blood and cord

blood of any mammal including human, preferably from the human bone marrow, wherein the age of the mammal does not limit the scope of the invention.

5 In order to increase the level of bHLH transcription factors in MSCs, it is preferred to transduce bHLH transcription factors into the MSCs directly, or using a suitable carrier, and it is most preferred to transduce a gene encoding a bHLH transcription factor or an active fragment thereof into the MSCs.

10 For introducing a bHLH transcription factor or an active gene fragment thereof into MSCs, the methods known in the art such as DNA-calcium precipitation method, the method using liposome or polyamines, electroporation and the method using retrovirus or adenovirus can be used. Especially, the method using retrovirus is preferred, and the bHLH transcription factor of the present invention can be transduced by a process
15 which comprises the steps of inserting the cDNA of a bHLH transcription factor into a retroviral vector to obtain an expression vector, transforming a packaging cell with said expression vector, culturing the transformed cell under an appropriate culture condition, filtering the culture medium to obtain a retroviral solution and transducing MSCs with the retroviral solution.
20 Then, MSCs which continuously express bHLH transcription factors can be prepared by using a selection marker present in the retroviral vector.

The MSCs transduced with the bHLH transcription factor of the present invention can differentiate to neuronal cells while inhibiting as osteogenic, chondrogenic, adipogenic and myogenic differentiation.
25 Therefore, MSCs can be differentiated into neuronal cells under a special *in vitro* condition and transdifferentiated into neuronal cells when the MSCs is transplanted into the brain of experimental animals. Conversion of the mesodermal potential into neural lineages can be confirmed by conducting *in vitro* experiments. For example, treatment of MSCs with forskolin and/or 5-
30 aza-deoxycytidin facilitate transdifferentiation of MSCs into neuronal cells.

In particular, in order to enhance the neuronal differentiation of MSC, it is preferred to add forskolin to MSCs to a final concentration of 10 to 30 $\mu\text{mol/l}$, and it is most preferred to pre-incubate MSCs with 5 to 30 $\mu\text{mol/l}$ of 5-aza-deoxycytidin for 3 to 10 days before adding forskolin.

35 Furthermore, the transdifferentiation efficiency can be increased by adding N2 supplement (Gibco) to the culture medium, preferably, the N2 supplement diluted 100 fold.

By conducting immunohistochemical analysis employing MAP2 (microtubul-associated protein 2), NF200 (neurofilament 200), or NeuN (neuronal nuclei) as a neuronal maker, it can be confirmed that the MSCs expressing bHLH transcription factors transplanted into the striatum of the experimental rats have transdifferentiated into neuronal cells to a much higher degree than MSCs which do not express transcription factors.

The MSCs transduced with a bHLH transcription factor or neuronal cells transdifferentiated therefrom can be used as an active ingredient of a pharmaceutical composition for cell therapy or gene therapy of neurological diseases such as Parkinson's disease, Alzheimer disease, Huntington's disease, amyotrophic lateral sclerosis, cerebral paralysis and brain ischemia; or for therapy of spine disfunction caused by an injury.

The neuronal cells which may be used in the pharmaceutical composition can be prepared by transducing one or more bHLH transcription factors into MSCs, propagating, and differentiating MSCs under a suitable condition *in vitro*; or by propagating MSCs, transducing a bHLH transcription factor into MSCs and differentiating transduced MSCs with a bHLH transcription factor.

For example, the desired gene for gene therapy of a specific brain disease (for example, the tyrosin hydroxylase gene involved in a synthesis of dopamine) can be introduced into the neurological system of an animal by transducing the viral vectors containing the desired gene into MSCs or the neuronal cells transdifferentiated therefrom, and transplanting the cells thus obtained into the brain of the animal. Therefore, the neuronal cells transdifferentiated from MSCs of the present invention can be used as a useful tool for delivering beneficial genes into the nervous system.

A pharmaceutical composition comprising the neuronal cells as an active ingredient can be injected into the patient's body according to the conventional methods well known in the art such as the clinical method disclosed in the open literature (Bjorklund and Stenevi, *Brain Res.*, 177, 555-560 (1979) and Lindvall et al., *Arch. Neurol.*, 46, 615-31(1989)).

The unit dose of the neuronal cells of the present invention to be actually administered ought to be determined in light of various relevant factors including the disease to be treated, the severity of the patient's symptom, the chosen route of administration, and the age, sex and body weight of the individual patient.

Moreover, the present invention also provides a kit for neuronal

differentiation of MSCs, which comprises the expression vector containing a bHLH transcription factor or an active fragment thereof.

The bHLH transcription factor which may be used in the present invention includes neurogenin 1, neurogenin 2, neuroD, MASH1, MATH3, E47 and an active fragment thereof; and the vector is preferably derived from retrovirus or adenovirus. The kit of the present invention may further comprise forskoline and/or 5-aza-deoxycytidin.

The following Examples are intended to further illustrate the present invention without limiting its scope; and the experimental methods used in the present invention can be practiced in accordance with Examples given herein below, unless otherwise stated.

Example 1 : Isolation and culture of mesenchymal stem cell

15

(step 1) Extraction of bone marrow and isolation of mesenchymal stem cells

4 ml of HISTOPAQUE 1077 (Sigma) and 4 ml of bone marrow obtained from Bone marrow bank (Korean Marrow Donor Program, KMDP) were added to a sterilized 5 ml test-tube. After centrifugation at 400 x g for 30 minutes, 0.5 ml of the buffy coat located in the interphase was collected and transferred into a test-tube containing 10 ml of sterilized phosphate buffered saline. The resulting suspension was centrifuged at 250 x g for 10 minutes to remove the supernatant and 10 ml of phosphate buffer was added thereto to obtain a suspension, which was centrifuged at 250 x g for 10 minutes. The above procedure was repeated twice and DMEM medium (Gibco) containing 10 % FBS (Gibco) was added to the resulting precipitate. A portion of the resulting solution corresponding to 1×10^7 cells was placed in a 100 mm dish and incubated at 37 °C for 4 hours while supplying 5 % CO₂ and 95% air. The supernatant was then removed and a new medium was added to continue culturing.

(Step 2) Culture and subculture of MSCs

The MSCs obtained in Step 1 were incubated using an MSC medium (10% FBS (Gibco) + 10 ng/ml of bFGF (Sigma) + 1% penicillin/streptomycin (Gibco) + 89% DMEM (Gibco)) in a CO₂ incubator kept at 37 °C. Serial incubations were carried out while changing the medium at an interval of 2 days. When the cells reached to 80% confluence,

the cells were collected using 0.25% trypsin/0.1mM EDTA (Gibco) and diluted 20 fold with the medium, and then, subcultured in the new dishes. The rest of cells thus obtained were kept frozen in a medium containing 10% DMSO (Sigma). Fig. 1 shows light microscopic photographs of
5 mesenchymal stem cells isolated from human bone marrow after cultivating for 1, 2, 3, and 7days *in vitro*.

(Step 3) Multiple differentiation potentials of MSCs

In vitro differentiation potentials of MSCs into adipocytes,
10 chondrocytes and osteocytes were examined as follows.

1) Adipogenic differentiation of hMSCs

MSCs were cultured in the MSC medium, followed by culturing in an adipogenic differentiation induction medium (DMEM medium (Gibco)
15 supplemented with 1 μ M dexamethasone (Sigma), 0.5 mM methylisobutylxanthine (Sigma), 10 μ g/ml of insulin (Gibco), 100 nM indomethacin (Sigma) and 10% FBS (Gibco)) for 48 hours. The resulting mixture was subsequently incubated in an adipogenic maintenance medium (DMEM medium containing 10 μ g/ml of insuline and 10% FBS) for 1 week
20 and stained with oil red O. As shown in Fig. 2, adipocytes stained with oil red O is pronounced inside the cells. This result suggests that MSCs can be successfully differentiated into adipocytes.

2) Chondrogenic differentiation of MSCs

25 MSCs were cultured in the MSC medium. 2×10^5 of the cells were collected using trypsin, centrifuged, and then, re-incubated in 0.5 ml of a serum-free chondrogenic differentiation induction medium (50 ml of high-glucose DMEM (Gibco) containing 0.5 ml of 100 x ITS (0.5 mg/ml of bovine insulin, 0.5 mg/ml of human transferrin, 0.5 μ g/ml of sodium
30 selenate (Sigma) and 10 ng/ml of TGF- β 1(Sigma)) for 3 weeks while replacing the medium every 3 days. Then, the cell masses were fixed with 4% paraformaldehyde, sectioned using a microtome, and then, stained with alcian blue (Fig. 2B). As shown in Fig. 2B, the extracellular cartilage matrix was stained blue and the presence of chondrocytes in cartilage
35 lacunae was observed. These results suggest that the MSCs were differentiated into chondrocytes.

3) Osteogenic differentiation of hMSCs

MSCs were incubated in the MSC medium, followed by culturing in 0.5 ml of an osteogenic differentiation induction medium (DMEM supplemented with 10 mM β -glycerol phosphate (Sigma), 0.2 mM ascorbate-2-phosphate (Sigma), 10 nM dexamethasone (Sigma) and 10% FBS (Gibco)) for 2 weeks while replacing the medium every 3 days. Then, the cells were fixed with 4% paraformaldehyde and stained for alkaline phosphatase and with von Kossa method. As shown in Figs. 2C and 2D, the increase of the alkaline phosphatase activity and the extracellular accumulation of calcium minerals in the form of hydroxyapatite suggest that the MSCs were differentiated into osteocytes.

Example 2 : Construction of a retroviral vector expressing neurogenin 1, a neurogenic transcription factor

(Step 1) Construction of retroviral vector expressing neurogenin 1 (ngn1)

PCR was carried out using oligonucleotides of SEQ ID NOS.: 1 and 2 as primers and genomic DNA of Balb/c mouse as a template, and the PCR product thus obtained was cloned using TOPO TA cloning kit (Invitrogen). Through sequence analysis of the vector cloned, it was confirmed that the vector contained neurogenin 1 gene. The neurogenin 1 cDNA was isolated with *EcoRI* and inserted into the *EcoRI* site of vector pMSCV-puro (Clontech, USA) with T4 DNA ligase (Roche) and transformed into *E. coli* DH5 α to obtain pMSCV-puro/ngn1. The resulting construct of pMSCV-puro/ngn1 is shown in Fig. 3.

(Step 2) Expression of neurogenin1 retroviral vector

pMSCV-puro/ngn1 vector was transfected into 293T/17 cell (ATCC CRL-11268) according to the calcium phosphate-coprecipitation method. After 48 hours of transduction, the cell was fractionated into cytoplasm and nucleus. In order to confirm the expression of neurogenin1, each fraction was subjected to western blot analysis using anti-neurogenin1 antibody (Chemicon). As shown in Fig. 4, neurogenin1 protein was expressed in the nuclear fraction of 293T/17 cell transfected with the pMSCV-puro/ngn1 vector.

(Step 3) Preparation of retrovirus containing neurogenin1

pMSCV-puro/ngn1 vector was transfected into retroviral packaging cell lines, PA317 (ATCC CRL-9078) or PG13 (ATCC CRL-10686) according to the calcium phosphate-coprecipitation method. After 48 hours of transduction, the culture solution was collected and filtered with 0.45 μ m membrane to obtain retrovirus solution. The retrovirus solution was kept at -70°C until use.

Example 3: Transduction of neurogenin1 into MSCs

10

MSCs were cultured to 70% confluence in 100 mm dishes. Added thereto was 4 ml of the retrovirus solution obtained in Example 2 which was mixed with polybrene (Sigma) to a final concentration of 8 $\mu\text{g/ml}$ and incubated for 8 hours. The retrovirus solution was then removed, the MSCs were cultured in 10 ml of MSC medium for 24 hours. The above procedure was repeated 1-4 times. Then, MSCs were collected using trypsin and diluted 20 fold with the medium. The obtained cells were subcultured in a medium supplemented with 2 $\mu\text{g/ml}$ of puromycin (Sigma) for 2 weeks. The puromycin positive cells were fractionated into cytoplasm and nucleus. In order to confirm the expression of neurogenin1, each fraction was subjected to western blot analysis using anti-neurogenin1 antibody. The result is shown in Fig. 5. As can be seen in Fig. 5, neurogenin1 protein was expressed in the nucleus of MSCs transduced with Ngn1 gene.

25 Example 4 : *In vitro* neuronal differentiation of MSCs expressing neurogenin1

(Step 1) Induction of neuronal differentiation

30 In order to induce *in vitro* neuronal differentiation of MSCs expressing neurogenin1 (MSC/Ngn1), 5×10^3 of the MSCs were plated on a cover-glass of 12 mm diameter coated with a mixture of poly-D-lysine and collagen (0.1 mg/ml each). Then, the cells were cultured in MSC medium supplemented with 10 $\mu\text{g/ml}$ of 5-aza-deoxycytidine (Sigma) for 3 to 7 days, followed by culturing in F12/DMEM supplemented with 10 μM of forskolin and N2 supplement (100 x, Gibco) for 1 week.

(Step 2) Immunocytochemistry

To examine neuronal differentiation of MSC/Ngn1, immunocytochemistry was carried out as follows.

Anti-MAP2 monoclonal antibody (Sigma, 1:200), Anti-NF200
5 polyclonal antibody (Sigma, 1:400) or Anti-NeuN monoclonal (Chemicon, 1:100) was used as a primary antibody, and a fluorescence-labeled, as a secondary antibody to detect the primary antibody according to the manufacturers' guidelines.

Cells were washed with PBS and fixed with 4% paraformaldehyde at
10 room temperature for 20 min. The fixed cells were washed three times with PBS and blocked with PBS containing 10% normal horse serum and 0.1% triton X-100 at room temperature for 2 hours. The cells thus obtained were reacted with the primary antibody overnight at 4 °C in PBS containing 10% normal horse serum, and washed three times with PBS containing 0.1%
15 Triton X-100 10 minutes each. The cells were added with the secondary antibody in PBS at room temperature for 1 hour and washed three times with PBS containing 0.1% Triton X-100 each for 10 minutes.

The result is shown in Fig. 6. As can be seen in Fig. 6, MSC/Ngn1 outgrew long neurites which were stained with antibodies against neurite
20 specific proteins, NF200 and MAP2. In addition, the cells were also immunoreactive to an antibody against neuronal nucleus specific NeuN antibody.

(Step 3) Electrophysiological recording

25 To examine functional maturation of the transdifferentiated neuronal cells, electrophysiological recording for the neuronal cell was carried out as follows.

The electric current of the cell membrane was amplified using an axopatch-1C patch-clamp amplifier (Axon Instrument, U. S. A.) and current
30 signals were filtered at 5 kHz to be displayed on an oscilloscope (20 MHz digital storage, type 1425, Gould, U. S. A.). The obtained current traces were analyzed with Clampfit v. 6.0.3. Physiological salt solution (PSS) was composed of 150mM NaCl, 5mM KCl, 1.5 mM CaCl₂, 1mM MgCl₂, 5mM glucose and 10 mM HEPES (N-(2-hydroxyethyl) piperazine N-(2-ethanesulfonic acid)). The pH of the physiological salt solution was
35 adjusted to 7.4 using NaOH and kept at 36 °C. Patch pipette was adjusted to 3-4MΩ and a Cs-rich pipette solution (consisting of 90mM Cs-aspartate,

55mM CsCl, 3mM Na₂ATP, 3 mM Na₂-creatine phosphate, 1mM MgCl₂, 10mM HEPES, 10mM EGTA, pH 7.4) was employed.

The result is shown in Fig 7. As can be seen in Fig. 7, an inward current was detected in 16 cells out of 27 MSC/Ngn1 cells (Fig. 7A). The inward current was selectively blocked by 4 μ M nicardipine which is an L-type Ca²⁺ channel blocker (Fig. 7B) and recovered by the removal of nicardipine (Fig. 7C). The current-voltage relation exhibited a bell shape typical of the voltage dependent L-type Ca²⁺ channel (Fig. 7D) but no current was measured in MSC/lacZ cells. The results suggest that Ngn1 induces the functional maturation of the neuronal cells by inducing the electrophysiological properties of human MSCs.

Example 5 : *In vitro* neuronal differentiation of MSCs co-expressing neurogenin1 and E47 or NeuroD1 and E47

To examine the effect the NeuroD1 gene, the neurogenin1 component of the expression vector obtained in Example 2 was replaced with NeuroD1 gene (Genbank Accession No. : U67776) and E47 (Genbank Accession No. : AF352579) to obtain NeuroD1 and E47 expression vectors, respectively. Mixtures of 4 μ g of neurogenin1 expression vector + 1 μ g of E47 expression vector, or 4 μ g of NeuroD1 expression vector + 1 μ g of E47 expression vector were mixed with 16 μ l of ExGEN500 (MBI Fermenta) and 1ml of 0.1 mM NaCl solution and added to 2 x 10⁵ of MSCs. Two hours after transfection, the medium was replaced with fresh MSC medium. Twenty-four hours after the medium was replaced with F12/DMEM supplemented with N2 supplement (100 x, Gibco) and waited 2 more days. In order to confirm the expressions of NF200, NF-M and β -tubulin III, neuronal cell markers, the resulting culture was subjected to western blot analysis using anti-NF200 (neurofilament 200, Chemicon), anti-NF-M (neurofilament M, Zymed), and anti- β -tubulin III (BAbCO) antibodies.

The result is shown in the Fig. 8. As shown in the Fig. 8, the expression of NF200 and NF-M proteins increased with the expression of neurogenin1/E47 or NeuroD/E47 in MSCs. The results suggest that the MSCs were transdifferentiated into neuronal cells. However, β -tubulin III was expressed in undifferentiated MSCs, and therefore, it failed to serve as an effective neuronal cell marker.

Example 6 : *In vivo* neuronal differentiation of MSCs expressing neurogenin1

(Step 1) Preparation of MSCs for transplantation.

5 MSCs expressing neurogenin1 were prepared according to the method of Example 3. In order to examine whether neurogenin1 gene increases the neuronal differentiation of MSCs, MSCs expressing β -galactoxidase (MSC/lacZ), which were to be used as a control, were prepared by carrying out the procedure of Example 2 except that Ngn1 gene
10 was replaced with lacZ gene. Before transplantation, MSCs expressing the lacZ or neurogenin1 gene were pre-stained with Hoechst 33258(Molecular Probe) or transduced with adenovirus carrying the nuclear localizing sequence bound to β -galactoxidase. The Hoechst 33258 pre-staining was carried out by incubating the cells in MSC medium supplemented with
15 1 μ g/ml of Hoechst 33258 (Molecular Probes) for 24 hours. The adenovirus transfection was carried out by adding the adenovirus solution (titer = 1×10^8 pfu/ml) with 100 m.o.i. (multiplicity of infection) for 3 hours. After pre-staining or transducing, MSCs expressing lacZ or neurogenin1 were collected using 0.25% trypsin/0.1% EDTA (Gibco) and diluted with
20 PBS (Gibco) to 3×10^3 cells/ μ l.

(Step 2) Transplantation

Transplantation was carried out using adult Sprague-Dawley albino rats as follows.

25 Female Sprague-Dawley albino rats were anesthetized by injecting a 350 mg/kg dose of chlroral hydrate (Sigma), the fur at the incision region was removed, and then, fixed to a stereotaxic frame. The vertex was sterilized with 70% ethanol and the 1 cm incision was made. After drilling at the exposed dura at positions of bregma + 1, ML + 3 and LV + 4, 1 μ l of
30 PBS containing 3×10^3 cells of MSC/lacZ or MSC/Ngn1 was injected at a rate of 0.2 μ l/min using a Hamilton syringe. Twenty minutes after injection, the syringe was removed. The incision was sutured. Cyclosporin A (Sigma), an immunosuppressant, was daily administered (5mg/kg) by intraperitoneal injection until the brain was extracted.

35

(Step 3) Preparation of tissue slice

Two and six weeks after transplantation, the rats were anesthetized

by injecting 400 mg/kg of chloral hydrate (Sigma). The rats were perfused with PBS and then with 0.1 M paraformaldehyde in PBS (pH 7.4). The brain was extracted and kept at 4 °C in 0.1 M paraformaldehyde in PBS (pH 7.4) for 16 hours to post-fix. The post-fixed brain was deposited in 30% sucrose for 24 hours and sectioned using a sliding microtome with a thickness of 35µm. The sections thus obtained were mounted to silane-coated slides (MUTO PUREW CHEMICAS CO., LTD, Japan) and stored at 4 °C in PBS till use.

Fig. 8 is a fluorescent microscopic photograph showing the integration rate of transplanted MSCs expressing lacZ or neurogenin1 gene in the striatum of the rats two weeks after transplantation. The graft efficiency is shown in Table 1.

Table 1

	Graft efficiency (%)
MSC	45
MSC/Ngn1	54

As can be seen in Fig. 9 and Table 1, the transplantation rate of MSC/Ngn1 was higher than that of MSC/lacZ. This result suggests that MSC expressing ngn1 was effective for cell therapy.

(Step 4) Immunohistochemistry

The tissue section mounted on a slide was kept in 1 x PBS/0.1% tritonX-100 for 30 minutes. To block non-specific interaction, the tissue section was reacted with 10% normal horse serum at room temperature for 1 hour, and then, reacted with a primary antibody at 4 °C for 16 hours. The primary antibodies were anti-NeuN mouse monoclonal antibody (Chemicon, Inc.) (1:200), anti-GFAP mouse monoclonal antibody (Sigma, Inc.) (1:200), anti-human nuclei antibody (Chemicon) (1:50) or anti-human Tau mouse monoclonal antibody (Abcam) (1:100). After washing three times with 1 x PBS/0.1% triton X-100 for 15 minutes, the sections were allowed to react with FITC-conjugated anti-mouse IgG (Vector, 1 : 200) or Texas red-conjugated anti-mouse IgG (Vector, 1: 200) to detect the primary antibody.

In order to further confirm the origin of the cells, a double immunohistochemistry analysis was carried out. The tissue section that were primarily stained with antibodies against NeuN, GFAP, and human

nuclei as above and then stained with anti- β -galactosidase rabbit polyclonal antibody (Abcam) (1:500) at room temperature for 2 hours, and then, reacted with Texas red-conjugated anti-rabbit IgG (Vector, 1: 200).

As can be seen in Figs. 10 and 11, MSCs expressing neurogenin1 were converted into neuronal cells with higher rates than MSC/lacZ. In case of MSC/lacZ, 45 % of the cells were positive for GFAP, a astocytic marker and 12% of the cells were positive for NeuN, a neuronal marker. In contrast, in case of MSC/Ngn1, 13 % of the cells were GFAP positive for GFAP, and 48% of the cells were NeuN positive (Fig. 12).

Six weeks after transplantation, MSCs expressing neurogenin1 were also transdifferentiated into NeuN-positive neuronal cells to a higher level than MSC/lacZ (Fig. 11). Through the double immunohistochemistry using anti-human nuclei mouse monoclonal antibody, it was confirmed that the cells reactive to anti- β -galactosidase rabbit polyclonal antibody were originated from the transplanted human cells (Fig. 13A). Furthermore, those cells were also positive for human Tau, a mature human neuronal cell maker. Therefore, it is confirmed that MSCs expressing neurogenin1 were transdifferentiated into mature neuronal cells (Fig. 13B).

This result suggests that the expression of neurogenin1 in MSCs induce the neuronal differentiation while inhibiting astroglial differentiation.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

What is claimed is :

1. A method for transdifferentiating mesenchymal stem cells into neuronal cells, which comprises increasing the level of a basic helix-loop-helix (bHLH) transcription factor in the mesenchymal stem cells.
2. The method of claim 1, wherein the bHLH transcription factor are selected from the group consisting of neurogenin 1, neurogenin 2, neuro D1, MASH1, MATH3, E47, a mixture thereof and active fragments thereof.
3. The method of claim 1 or 2, wherein the step of increasing the level of the basic helix-loop-helix (bHLH) transcription factor in the mesenchymal stem cells is carried out by transducing the bHLH transcription factor or an active fragment thereof into MSCs and expressing same.
4. The method of claim 3, wherein the step of transducing the bHLH transcription factor or active fragment thereof into the mesenchymal stem cells is conducted by transducing the mesenchymal stem cells with a viral vector encoding the bHLH transcription factor or active fragment thereof.
5. The method of claim 1, which further comprises the step of adding forskolin, 5-azadeoxycytidin or a mixture thereof to the mesenchymal stem cells.
6. The method of claim 5, wherein forskolin is added in an amount of 10 to 30 $\mu\text{mol/l}$.
7. The method of claim 5, wherein 5-aza-deoxycytidin is added in an amount of 5 to 30 $\mu\text{mol/l}$ before 3 to 10 days of forskolin addition.
8. The method of claim 1, which comprises culturing the mesenchymal stem cells in a medium supplemented with N2 supplement.
9. A pharmaceutical composition for cell therapy of a neurological disease, which comprises a therapeutically effective amount of the mesenchymal stem cells transduced with a bHLH transcription factor or the neuronal cells transdifferentiated therefrom.

10. The pharmaceutical composition of claim 9, wherein the neurological disease is Parkinson's disease, Alzheimer disease, Huntington's disease, amyotrophic lateral sclerosis, cerebral paralysis, brain ischemia or spine dysfunction caused by a traumatic injury.

11. The pharmaceutical composition of claim 9, wherein the neuronal cells are prepared by transducing the bHLH transcription factor into the mesenchymal stem cells and propagating or differentiating the mesenchymal stem cells under a suitable *in vitro* condition; or by propagating the mesenchymal stem cells, transducing the bHLH transcription factor into the mesenchymal stem cells and differentiating the transduced mesenchymal stem cells.

12. A kit for transdifferentiating mesenchymal stem cells into the neuronal cells, which comprises an expression vector encoding a bHLH transcription factor or an active fragment thereof.

13. The kit of claim 12, wherein the bHLH transcription factor is selected from the group consisting of neurogenin 1, neurogenin 2, neuroD1, MASH1, MATH3 and E47.

14. The kit of claim 12 or 13, which further comprises forskolin or 5-aza-deoxycytidin.

THIS PAGE BLANK (U)

1/8

Fig. 1

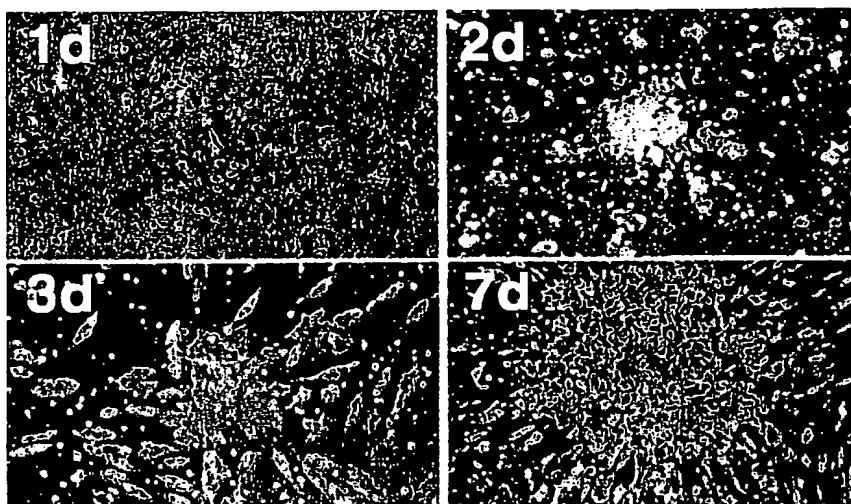
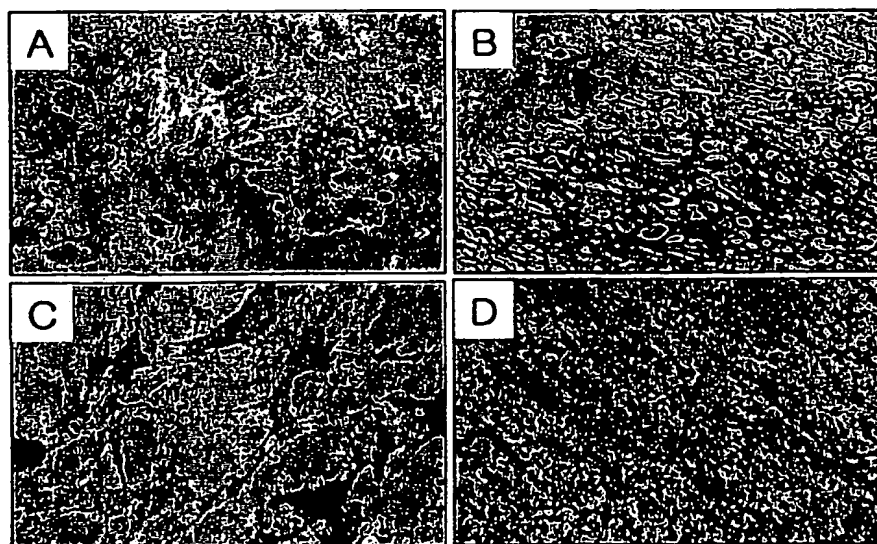


Fig. 2



THIS PAGE **BLANK** (USPTO)

2/8

Fig. 3

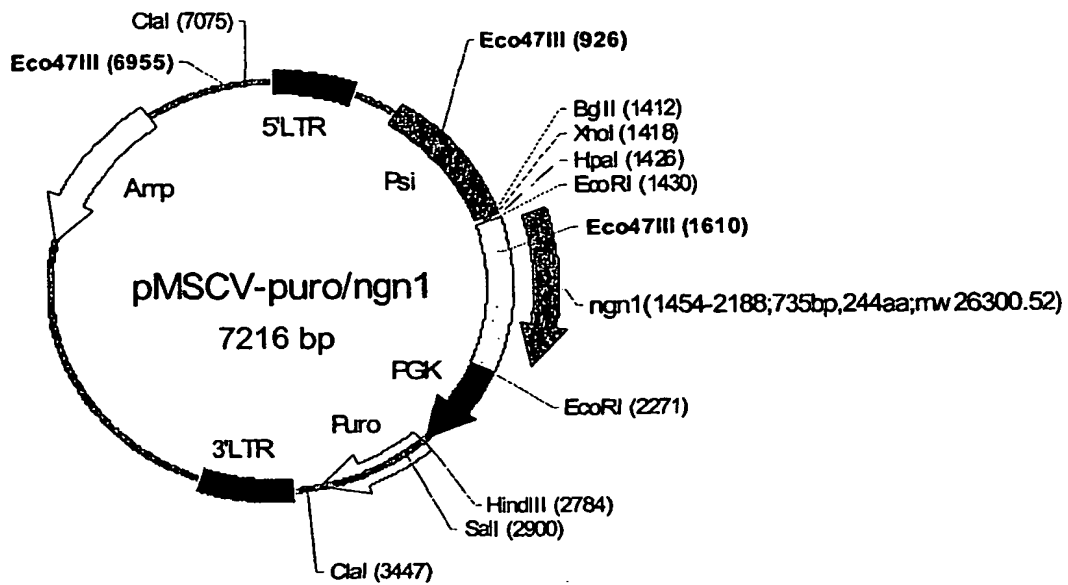


Fig. 4

	293T		293T/Ngn1	
	Cyt.	Nu.	Cyt.	Nu.
Neurogenin 1				

THIS PAGE BLANK

3/8

Fig. 5

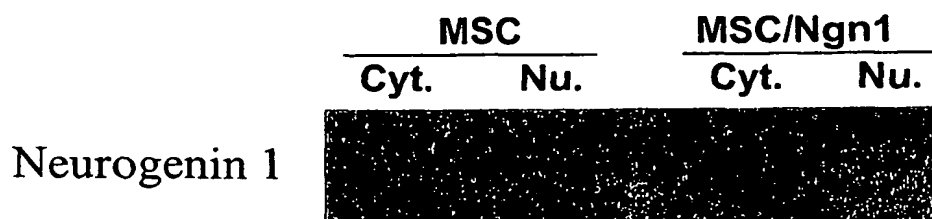
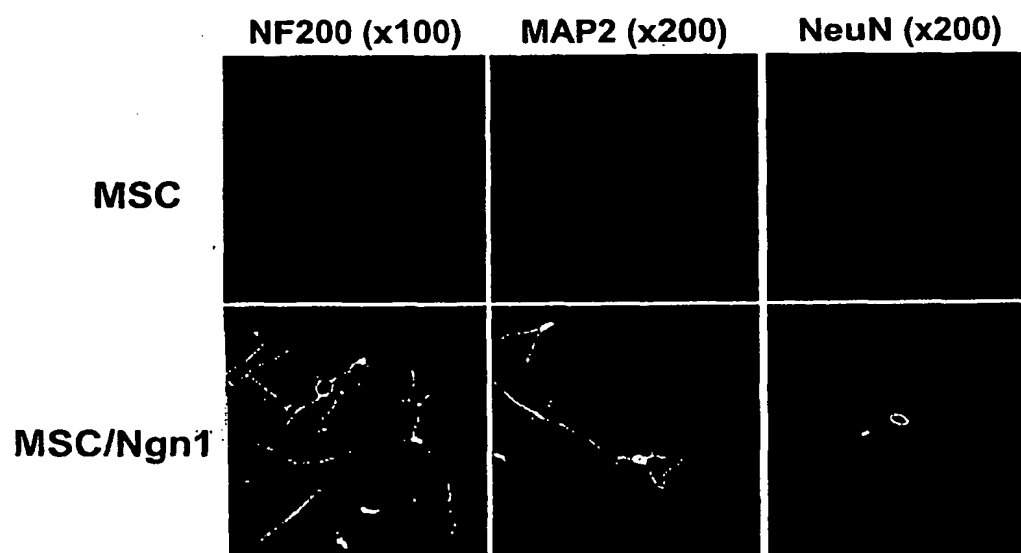


Fig. 6

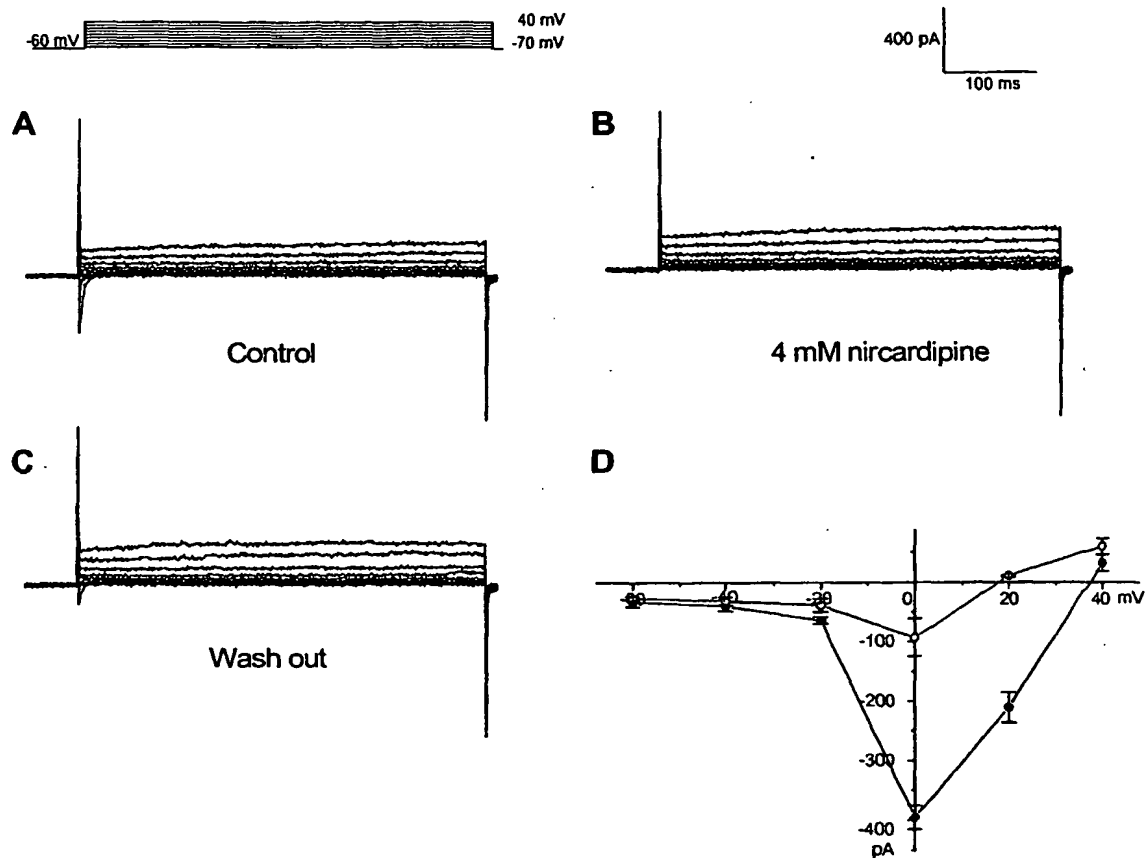


DTIC Rec'd PGT/PTO 17 FEB 2005

THIS PAGE BLANK (U.)

4/8

Fig. 7



DT12 Rec'd PCT/PTO 17 FEB 2005

THIS PAGE BLANK (UC)

5/8

Fig. 8

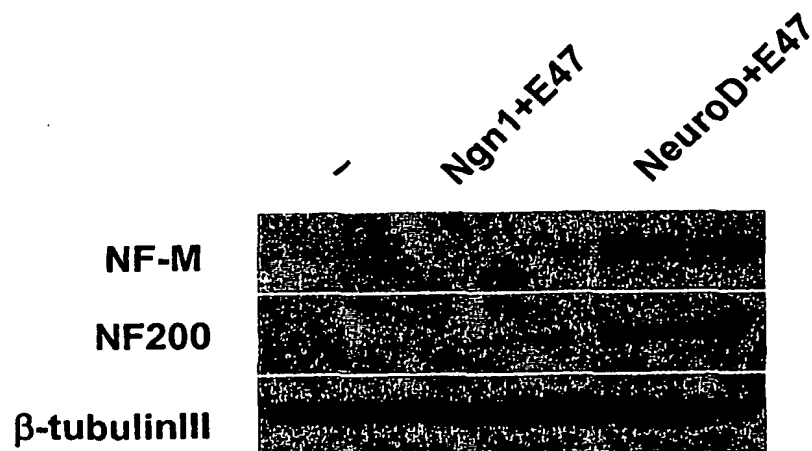
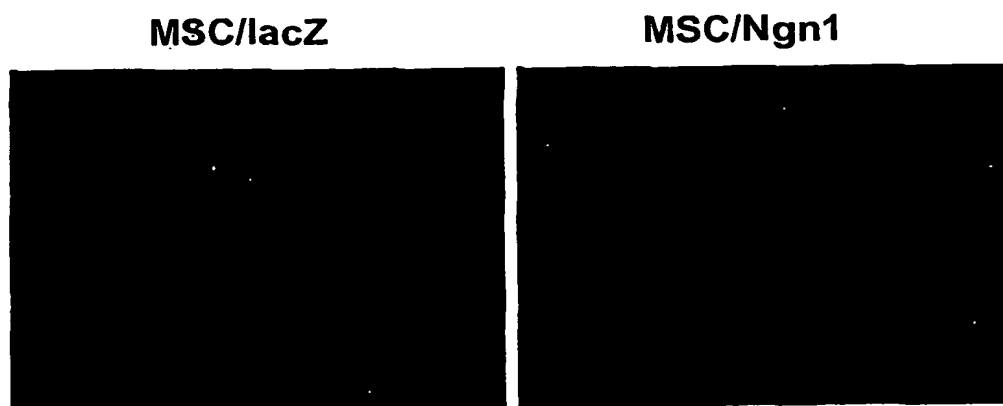


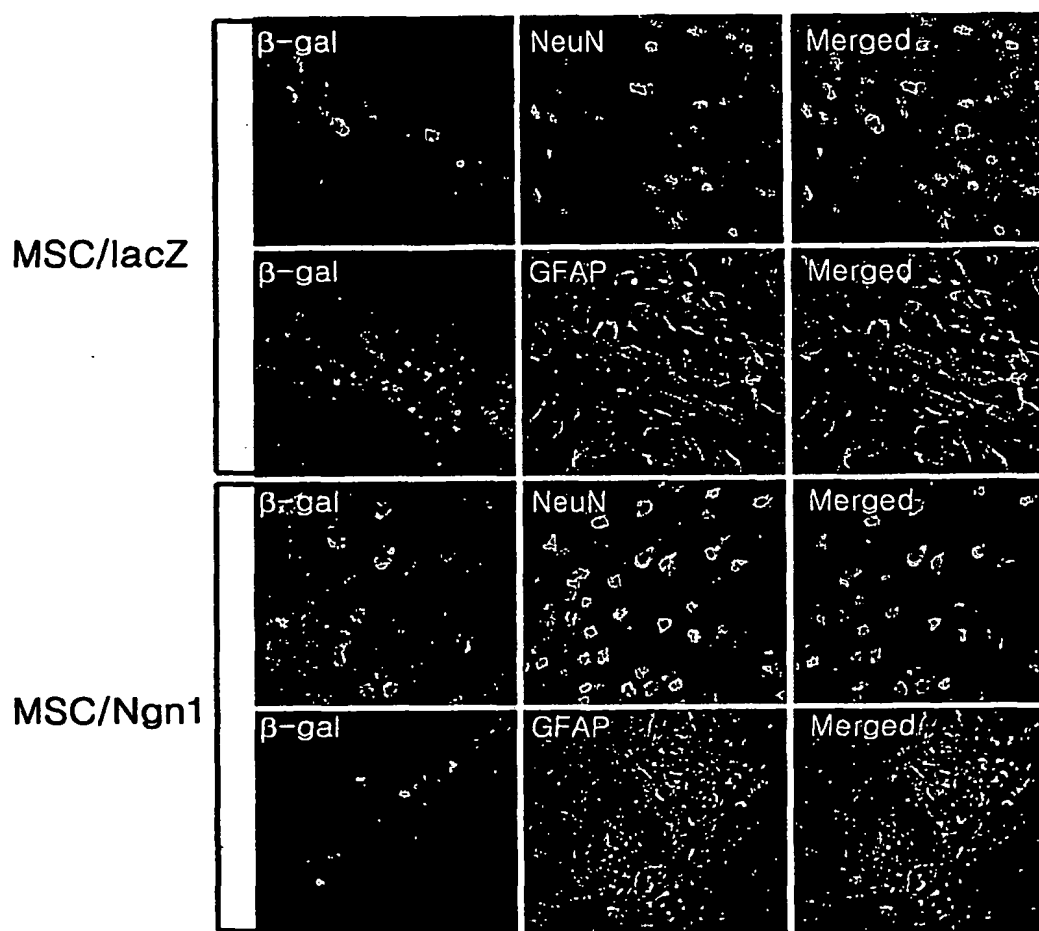
Fig. 9



THIS PAGE BLANK (UE

6/8

Fig. 10



THIS PAGE BLANK (USPTO)

7/8

Fig. 11

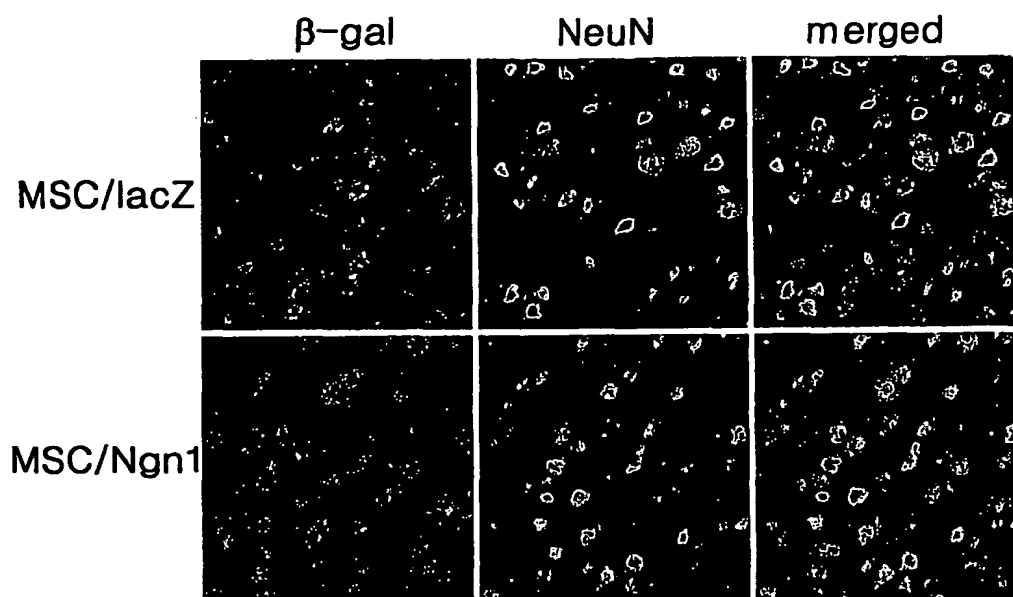
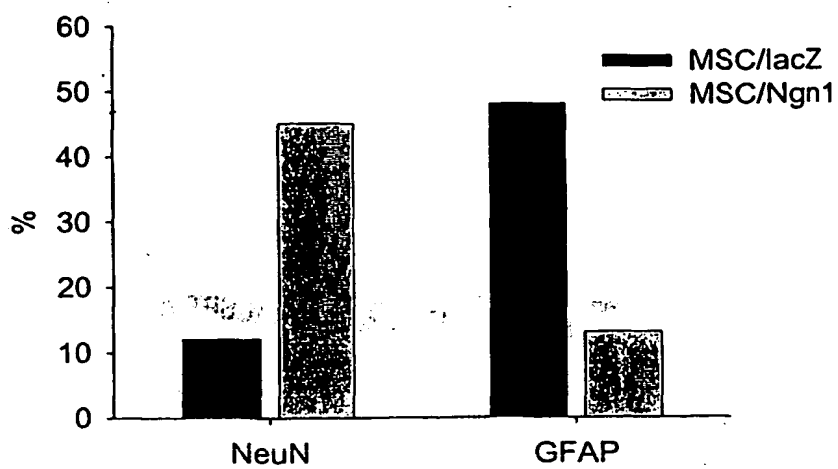


Fig. 12

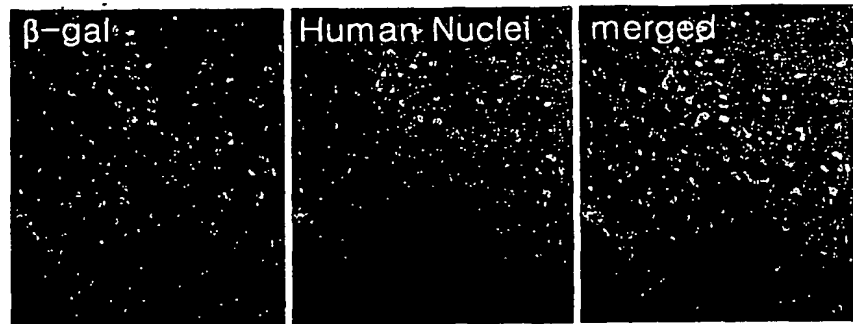


THIS PAGE BLANK (USPTO)

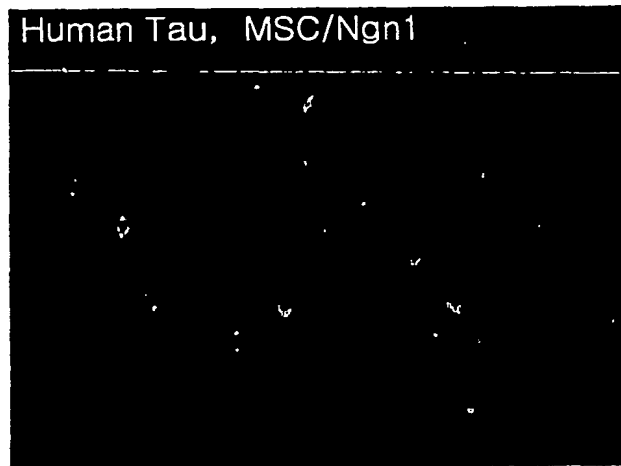
8/8

Fig. 13

(A)



(B)



DT12 Rec'd PCT/PTO 17 FEB 2005

THIS PAGE BLANK (USPTO)

Sequence Listing

<110> SUH, HAE YOUNG

<120> A METHOD FOR TRANSDIFFERENTIATING MESENCHYMAL STEM CELLS INTO
NEURONAL CELLS

<150> KR 10-2002-48683

<151> 2002-08-17

<160> 2

<170> KopatentIn 1.71

<210> 1

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> primer for Neurogenin 1

<400> 1

tgcaagatcc tgccccttt

19

<210> 2

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> primer for neurogenin 1

<400> 2

tctaccctt ctggctgaag c

21

THIS PAGE BLANK (USPTO)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01641**A. CLASSIFICATION OF SUBJECT MATTER**

IPC7 C12N 15/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/08, C12Q, C12N 5/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean patents and applications for inventions since 1975Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Kipass, PubMed, Delphion, CA, PAJ "mesenchymal stem cell", "bHLH"**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,942,225 A(Case Western Reserve University) 24 August 1999 see the abstract	1-14
A	US 5,795,723 A(Fred Hutchinson Cancer Research Center) 18 August 1998 see the abstract	1-14
A	Kazhdan I et al, "HLH transcription factor activity in osteogenic cells" J Cell Biochem 1997 Apr;65(1):1-10 see the abstract	1-14
A	Zhao LR et al, "Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats" Exp Neurol 2002 Mar;174(1):11-20 see the abstract	1-14
P,A	WO0286108 A(LIFECORD INC.) 31 October 2002 see the whole documents	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family


Date of the actual completion of the international search

29 DECEMBER 2003 (29.12.2003)

Date of mailing of the international search report

29 DECEMBER 2003 (29.12.2003)

Name and mailing address of the ISA/KR

 Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

KIM, Jae Hyun

Telephone No. 82-42-481-5591



THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)